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Behavioral and Biochemical Effects of Early Postnatal Cholinergic Lesion in the Hippocampus

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GÁSPÁR, E., M. HEERINGA, É. MARKEL, P. G. M. LUITEN AND C. NYAKAS. *Behavioral and biochemical effects of early postnatal cholinergic lesion in the hippocampus*. BRAIN RES BULL 28(1) 65–71, 1992.—The effects of early postnatal (PD 8) intracerebroventricular injection of ethylcholine mustard aziridinium ion (AF64A) on development of open-field and cognitive behaviors and cholinergic markers in several brain areas were examined in the rat. The cholinotoxin was bilaterally administered in a dose range of 0.25 to 2.0 nmol. In the open-field tests, the cholinergic lesion caused a dose-dependent increase in activity at 20 days of age, while it resulted in lengthened latency to initiate exploration and decreased rearing activity at adulthood. Hole-board spatial learning was severely inhibited in adult age. The biochemical activity of choline acetyltransferase (ChAT) and acetylcholinesterase (AChE) in the hippocampus was markedly decreased in a dose-dependent manner, but was unchanged in the neocortex and striatum. Histochemical staining of AChE-positive fibers revealed a severe cholinergic denervation of the granular and pyramidal cell layers of the hippocampus. The results showed that a selective cholinergic deafferentation of the hippocampus at a critical stage of development leads to long-lasting abnormal open-field and spatial learning behaviors.

AF64A	Development	Hippocampus	Cholinergic markers	Open-field activity	Spatial learning
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A number of perinatal risk factors, such as hypoxia/anoxia (16, 29, 42), ischemia (34) or ethanol exposure (38) have been shown to suppress or interfere with the hippocampal and neocortical cholinergic systems in the developing brain. Behavioral teratology research has provided ample evidence showing that the same risk factors induce a permanent deterioration of cognitive functions. Our own studies have also revealed long-lasting effects of perinatal hypoxia and anoxia on adult cognitive and motivational behaviors, and a concomitant developmental suppression of the cholinergic system in cortex and hippocampus (29–31). Since the effects of hypoxia or other risk factors on the central nervous system are not specific to the cholinergic system, although this transmitter system appears particularly sensitive to hypoxia (40), it became interesting to study the behavioral effects of a selective cholinergic forebrain lesion performed during postnatal development.

The innervation of archi- and neocortex by cholinergic fibers of subcortical origin occurs relatively late during ontogenesis (36) as compared to that of subcortical brain structures themselves. According to previous studies on the development of the septo-hippocampal cholinergic projection, the activities of the marker enzymes choline acetyltransferase (ChAT) and acetylcholine esterase (AChE) and the high-affinity choline uptake appear around postnatal days 3 to 4 (25–27, 39). In the present study, we attempted to lesion the forebrain cholinergic neurons by the cholinotoxin ethylcholine mustard aziridinium ion (AF64A), applied at postnatal day 8 (PD 8). At this time of development,

the ingrowing cholinergic cortical afferents are already provided with functionally active nerve endings, and offer a neural substrate for the toxin to interfere with high-affinity choline transport and with the synthesis of acetylcholine (20).

AF64A has been considered to act as a potent cholinotoxin by inhibiting transmembrane choline transport in the presynaptic terminals of cholinergic afferents, thus causing their degeneration (11,35). Furthermore, it inhibits the acetylcholine biosynthetic enzyme ChAT (11,37). The intracerebroventricular (ICV) application of AF64A has been frequently used in model experiments aimed to selectively destroy brain cholinergic structures (10).

A number of studies have reported on the deleterious effects of cholinergic lesions with AF64A on learning and memory functions in adult rats (1, 8, 13, 28, 43), but only rarely in developing animals (41). It has been pointed out that AF64A selectively destroys presynaptic cholinergic terminals when given in a proper low dose (12, 13, 33). In these latter experiments, the most extensive cholinergic lesion was found in the hippocampus, whereas other structures like neocortex or striatum were less influenced by the toxin (17,21). Thus a second purpose of the present investigation was to gain insight in the region-specific and dose-dependent effects of early postnatal administration of AF64A in relation to behavioral impairments. For this reason, we measured the activity of cholinergic marker enzymes (ChAT and AChE) in three brain regions: hippocampus, parietal cortex and striatum, after a dose of 2.0 to 0.25 nmol of AF64A

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was injected into the lateral ventricles. To assess the behavioral effects of the toxin, the novelty-induced exploratory activity and the hole-board spatial learning tests were selected. Both the spontaneous behavioral activation in a novel environment and the spatial learning ability are strongly dependent on intact septo-hippocampal function (18). To study a possible recovery in the cholinergic hippocampal afferentation after the early post-natal lesion, the activity of ChAT and AChE were compared after measurements at PD 20 and at adult age.

METHOD

Animals and AF64A Treatment

In total, 122 male offspring of Wistar rats bred in our laboratory were used. Special care was taken that all individuals that formed a group were selected from different nests. At the age of 8 days, the pups were randomly assigned to five groups and injected bilaterally into the lateral cerebral ventricles (ICV) either with different doses of AF64A (0.25, 0.5, 1.0, and 2.0 nmol, experimental groups) or with the vehicle solution of 2 μ l buffered physiological saline (sham-lesioned group). The acetylthiocholine mustard-HCl (RBI, Natick, USA) was subjected to basic hydrolysis and subsequent formation of the aziridinium ion as described earlier (12,33). The ICV infusion of the toxin or vehicle solution was carried out under ether anesthesia and lasted for 10 min. The tip of a Hamilton injection needle was aimed at the lateral ventricles at stereotaxic coordinates relative to bregma: AP 0.0, L \pm 1.4, and V 4.0 (measured from the surface of the skin). The pups were randomly distributed among the mother rats so that each mother nursed 7 to 8 pups. The behavioral tests were performed on 20-day- and on three-month-old animals. One to two weeks after the last testing in adult age, the rats were sacrificed for biochemical and histological examination.

Behavioral Procedures

Open-field activity. In 20-day-old rats the novelty-induced behavioral activity was assessed in a small open field (SOF), which was a 30-cm high glass cylinder with a diameter of 16 cm. Six rats were observed simultaneously and the intensity of exploration-like activity was estimated by a time-sampling method. The behavior was scored every 10 s throughout a 10-min observation period (totally 60 observations) according to the following arbitrary scale: 0—immobility, 1—sniffing with head movements, 2—walking, and 4—rearing. Adding up the scores served to express novelty-induced activity. Grooming was recorded separately when movements such as face washing or body grooming occurred. Upon the rats' reaching the age of 3 months, open-field activity was measured again in a cylindrical arena of 80 cm in diameter surrounded by a 35-cm wall in a three-minute test (31). The arena was divided into 20 sectors and lighted from above by a 60-W bulb. The following behavioral items were distinguished: a) latency of leaving the center of the arena, b) ambulation, i.e., number of line crossings between sectors, c) number and duration (s) of rearings, and d) duration of grooming episodes in seconds.

Spatial learning in a hole-board test. At the age of 3 months, the animals were tested in a hole board (32) to study food-motivated spatial learning behavior. A rectangular arena with 35-cm high walls contained 16 equidistant holes, 13 cm apart. Beginning three days before testing, the rats were food deprived by feeding them 5 g food per day in their home cage. The loss in body weight was recorded by daily weight measurements. On days 4 to 8, the rats were habituated to the experimental box for 20 min and consumed their food in the test arena. All 16 holes

contained a food pellet of about 40 mg. After each session, the animals received a complementary portion of the same standard chow in the home cage in order to maintain their body weight around the level of 90% of the original body weight. In the learning phase of the test from day 9 to day 13, food was offered only in 4 out of 16 holes applied in a fixed spatial pattern, i.e., the same 4 holes were baited throughout all five sessions, while food pellets were covered in the unbaited (nonfood) holes. Each daily session consisted of 10 trials with an intertrial interval of 30–40 seconds. During trials, first the rats were placed in a start box. After opening the door of the start box, the rat entered the arena, and the number of visited food holes (FH) and nonfood holes (NFH) were counted until all FH were emptied. Learning performance was expressed as a ratio of FH/FH + NFH as described previously (32).

Neurochemical Determinations

The animals were sacrificed at PD 20 or at the age of 3 months. To avoid blood contamination of brain tissue, the rats were transcardially perfused with heparinized saline under pentobarbital anesthesia. The brain was rapidly removed from the skull, and several brain areas were dissected out macroscopically. The activity of two cholinergic marker enzymes, choline acetyltransferase (ChAT) and acetylcholinesterase (AChE), was determined in hippocampus, parietal neocortex, and corpus striatum. For ChAT determination, the method described by Brownstein et al. (4) was followed. Briefly, the tissue samples were homogenized in 10 vol. 0.1 M phosphate buffer, pH 6.0. The final incubation volume of 40 μ l contained 0.25 M NaCl, 0.1 mM neostigmine, 12.5 mM choline chloride, 0.1% (v/v) Triton-X-100, and 1.25 mM 14 C-acetyl coenzyme A (5 mCi/mmol, Amersham). After incubation at 37°C for 30 min, the samples were passed through DOWEX 1 (HCO_3^- , Serva, Heidelberg) for elimination of labeled acetyl coenzyme A. The activity of the enzyme was expressed in μ mol acetylcholine synthesized per hour per 1 g protein of the sample. The activity of AChE was assayed with the colorimetric method of Ellman et al. (9). The tissue samples were homogenized in 50 vol. 0.1 M phosphate buffer, pH 8.0, containing 1% Triton-X-100. The enzyme activity was expressed in μ mol/min/g protein. For protein determination, the method of Lowry et al. (22) was used.

Acetylcholinesterase Histochemistry

The AChE-positive fibers were stained according to a modified AChE-histochemical procedure (15). Five adult rats injected with 2.0 nmol AF64A and two sham-lesioned controls were transcardially perfused with 2.5% glutaraldehyde in 0.05 M PB, pH 7.4, the brains removed and serially sectioned with a cryostat microtome. The 10- μ m thick free-floating sections were stained with a procedure including reactions in subsequent steps of acetylthiocholine, sodium sulfide, sodium nitrate, and silver nitrate, yielding a clear staining pattern of AChE positive fibers.

Statistics

The data were analyzed with ANOVA according to the STATS program. Post hoc comparisons between two groups were performed by the Dunnett test. For statistical analysis of neurochemical data, Student's *t*-test was applied when only two groups were run in the experiment.

RESULTS

Open-Field Activity

The AF64A treatment profoundly changed open-field activity of both 20-day-old (Fig. 1) and 3-month-old animals (Fig. 2).

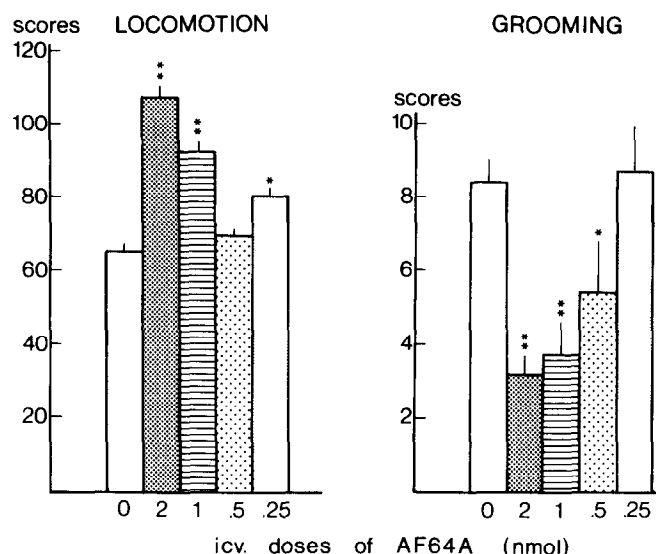


FIG. 1. Open-field activity of 20-day-old rats exposed to different doses of AF64A at postnatal day 8. Means \pm SEMs of combined scores of locomotion and the occurrence of grooming activity are shown. The number of animals varied between 10 and 15. * $p < 0.05$, † $p < 0.01$ versus vehicle-injected control (0 nmol, Dunnett *t*-test).

One-way analysis of variance revealed that exploration-like locomotion was increased by the treatment in the young rats [Fig. 1, $F(4,60) = 19.42$, $p < 0.001$], while grooming decreased in a dose-dependent manner, $F(4,60) = 10.16$, $p < 0.001$. The significant differences between the paired treated and control groups are indicated by asterisks in Fig. 1. The lower doses only slightly influenced the novelty-induced behavioral activities, but the 1 and 2 nmol doses resulted in marked changes. As an overall effect, the AF64A treatment led to a hyperactivity since an

increment in locomotion dominated the behavior. In fact, the increased locomotion coupled with decreased grooming reflected a shift within these measures of behavioral arousal in favour of the former in the course of 10-min test period.

Results obtained in adult rats (Fig. 2) show that the latency to start exploration was markedly increased in rats treated with the neurotoxin [one-way ANOVA, effect of treatment is significant: $F(4,30) = 11.27$, $p < 0.001$], while measures of rearing activity were decreased, $F(4,30) = 17.04$, $p < 0.001$. In both behavioral items, only the higher doses were effective (1 and 2 nmol). The line-crossing activity was not significantly influenced, $F(4,30) = 1.94$, $p = 0.13$. Grooming observed during this 3-min test was only slightly modulated by the treatment, $F(4,30) = 2.14$, $p = 0.10$. Less grooming occurred in rats treated with the 1 nmol dose ($t = 1.82$, $p < 0.05$, one-tailed *t*-test).

Spatial Learning in a Hole-Board Test

As illustrated in Fig. 3, the early postnatal AF64A treatment markedly influenced the learning performance of adult rats, as revealed by a two-factor ANOVA with repeated measures on the session factor, $F(4,41) = 65.27$, $p < 0.001$. In the course of 5 sessions, there was a clear improvement in the learning process [significant effect of sessions: $F(4,164) = 242.04$, $p < 0.001$]. The interaction between the two main effects (treatment \times sessions) was also significant, $F(4,164) = 41.16$, $p < 0.001$, indicating that the rate of improving performance from session to session was retarded by the AF64A treatment. The neurotoxin impaired spatial learning in a dose-dependent manner, based on a significant treatment effect among the AF64A-treated groups, $F(3,28) = 53.67$, $p < 0.001$. The inhibitory action of higher doses of 2 and 1 nmol was clearly present in each session (significant differences yielded by post hoc paired comparisons are shown in Fig. 3). The rats treated with the 0.5 nmol dose showed a performance comparable to controls except at session 5 ($p < 0.05$, Fig. 3). The lowest dose used (0.25 nmol) did not influence the spatial learning behavior of rats. Although treated rats performed this task worse than controls, they showed an improvement from

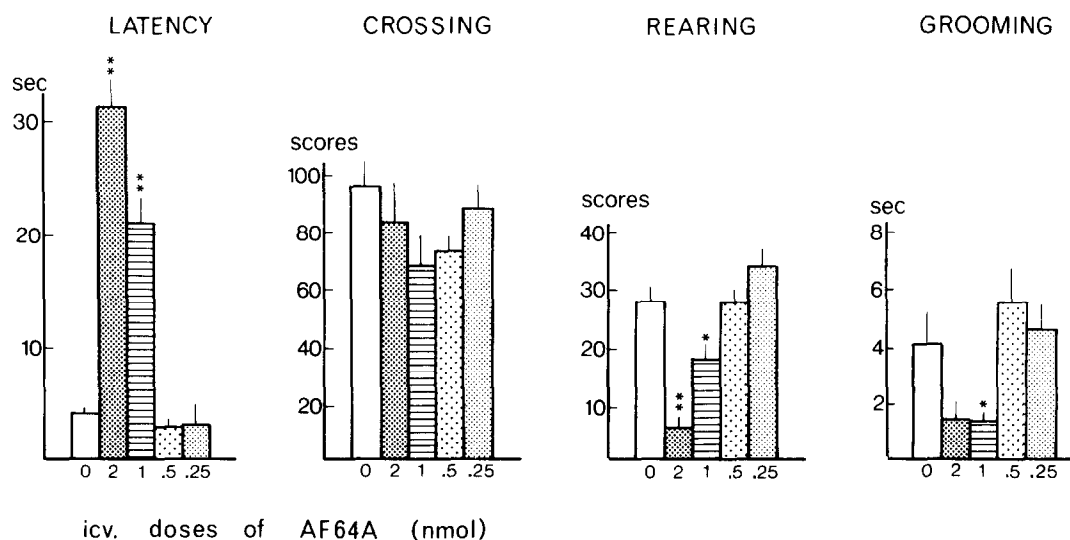


FIG. 2. Open-field activity of adult rats (3 months) subjected to the early postnatal cholinergic lesion with different doses of AF64A. Latency to start ambulation, the number of line crossings in the arena, the number and duration of rearing, and the time spent with grooming are shown by means \pm SEMs. * $p < 0.05$, † $p < 0.01$ versus vehicle-infused control group (0 nmol).

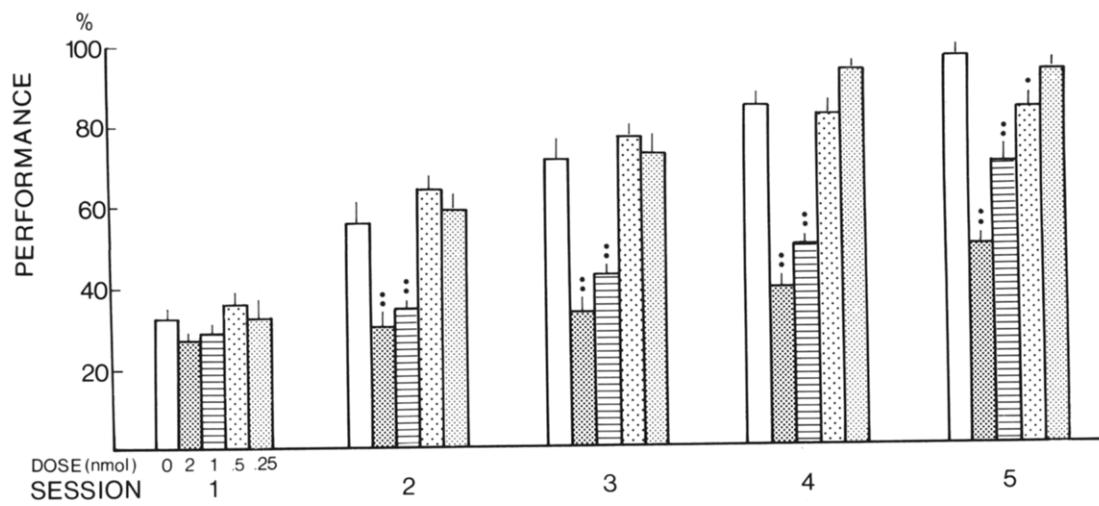


FIG. 3. Learning performance of adult rats in a hole-board spatial test after early postnatal AF64A infusion into the lateral cerebral ventricles. The different doses in nmol are indicated under the first group of columns. Groups contained 7 to 11 animals. * $p < 0.05$, † $p < 0.01$ versus vehicle (0 nmol) controls.

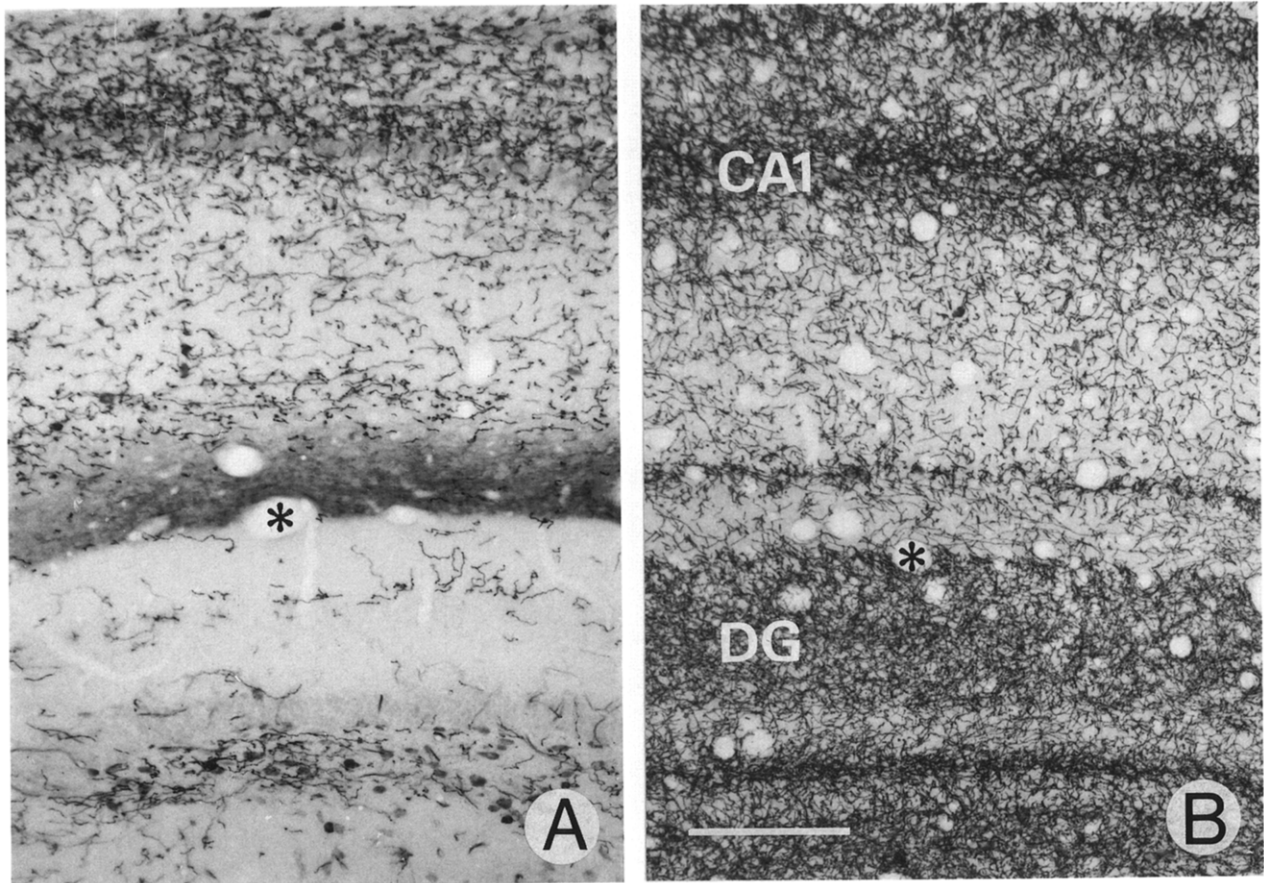


FIG. 4. Photomicrographs of AChE-stained fibers in the dorsal hippocampus in a 2 nmol injected AF64A case (A) and a control case (B). Cornu ammonis field 1 (CA1) and dentate gyrus (DG) are separated by the fissura hippocampi labeled by asterisks. Scale bar = 50 μ m.

TABLE 1
EFFECT OF INFANTILE ICV ADMINISTRATION OF AF64A ON ChAT AND AChE ENZYME
ACTIVITIES IN SEVERAL BRAIN REGIONS

	Doses (nmol)	ChAT ($\mu\text{mol/h/g pr}$)	%	AChE ($\mu\text{mol/min/g pr}$)	%
Hippocampus					
20 days	0	26.10 \pm 0.75	100	54.1 \pm 5.6	100
	2.0	5.53 \pm 0.36‡	21	26.4 \pm 4.8‡	49
Adult	0	47.88 \pm 3.60	100	50.5 \pm 4.3	100
	0.25	41.42 \pm 7.76	87	nm	
	0.5	34.85 \pm 4.36*	73	nm	
	1.0	30.91 \pm 1.69†	65	nm	
	2.0	20.53 \pm 4.00‡	43	20.1 \pm 3.1‡	40
Neocortex					
20 days	0	19.64 \pm 1.32	100	25.2 \pm 1.1	100
	2.0	19.49 \pm 0.49	99	24.4 \pm 1.5	97
Adult	0	28.64 \pm 1.84	100	28.3 \pm 4.4	100
	2.0	28.18 \pm 1.96	98	21.8 \pm 1.4	77
Striatum					
20 days	0	75.19 \pm 2.86	100	274.6 \pm 10.5	100
	2.0	85.00 \pm 3.79	113	313.4 \pm 31.7	114
Adult	0	139.62 \pm 5.49	100	262.5 \pm 17.5	100
	2.0	137.72 \pm 6.74	99	278.8 \pm 22.1	106

Values are means \pm SEMs from 5 to 8 animals per group. * p <0.05, † p <0.01, ‡ p <0.001 vs. control (0 nmol). nm: not measured.

session to session, even after the 2 nmol dose [significant effect of sessions: $F(4,36)=22.45$, $p<0.001$]. Therefore, the learning impairment was only relative to controls.

Cholinergic Markers

The biochemical activity of enzymes ChAT and AChE in the hippocampus was considerably lower after AF64A treatment (Table 1). The enzymatic activities did not change in the other two regions examined, i.e., in the neocortex or in the striatum. The toxin decreased hippocampal ChAT activity in a dose-dependent manner, as is shown in the adult rats. The decrease caused by the two higher doses of 1 and 2 nmol was statistically highly significant ($p<0.01$, Dunnett t -test). The 0.5 nmol dose was the lowest which significantly reduced ChAT activity ($p<0.05$). There were no age-dependent differences in regional AChE activities in the control groups. The ChAT activity in the controls, however, was lower in the 20-day-old than in the adult hippocampus [$t(13)=5.92$, $p<0.001$, Student's t -test], but also in the neocortex, $t(10)=3.98$, $p<0.01$, and striatum, $t(11)=10.4$, $p<0.001$. The proportional loss of ChAT activities in the hippocampus after the 2 nmol AF64A injection was about the same in the two age groups (no significant interaction between age and treatment, $p=0.79$). The lack of any significant recovery can be also demonstrated by the values of AChE enzyme activities if the percent changes are compared in the 20-day-old and adult animals.

Histological examination of hippocampal cholinergic afferentation by AChE staining showed a very substantial loss of fibers in all areas and layers (Fig. 4). The disappearance of fibers was most pronounced in the molecular and granular layers of the dentate gyrus (DG). The strata radiatum and moleculare of the

cornu ammonis (CA) regions and the hilus of DG were less severely denervated. Innervation of the stratum oriens was, relatively speaking, better preserved. Also, interneurons in stratum oriens and in the hilus showed more intense AChE-positive staining, and more neurons became visible with this staining. The dorsal hippocampus showed more extensive loss of AChE-positive fibers than the ventral hippocampus. Occasionally, also nonspecific tissue damage was observed in the CA3 area in the vicinity of lateral ventricles.

DISCUSSION

Early postnatal ICV administration of the cholinotoxin AF64A resulted in a selective and long-lasting lesion of hippocampal cholinergic afferentation. The activities of the cholinergic marker enzymes ChAT and AChE were dose-dependently decreased in the hippocampus but were not significantly influenced in the neocortex and in the corpus striatum. The density of histochemically stained cholinergic fibers was reduced mainly in the layers that contain the apical dendrites and perikarya of the hippocampal principal neurons. The AChE fiber staining also disclosed that the dentate gyrus was more severely denervated than CA regions. The stratum oriens and the hilus containing more passing fibers and interneurons, respectively, were relatively spared by the toxin treatment. It should be noted that the neurochemical specificity of AF64A towards the cholinergic system is not without controversy in the literature (7,19). Even a limited extent of tissue necrosis might lesion cholinergic as well as other passing fibers. In the present study, ventricular dilatation with a limited lesion in the CA3 region was occasionally observed. This lesion was asymmetrical and restricted to either side of the brain. The different aspects of region-selective destruction of AChE fi-

bers discussed above, however, support a rather selective action of the toxin in the early postnatal period. Finally, there was no significant recovery in cholinergic enzyme activities during the developmental period from PD 20 to adult age, which indicates a minor, if any, capacity for homotypic regeneration.

Rats subjected to AF64A lesions at the age of 8 days showed abnormal novelty-induced behavior. In the preweaning age (PD 20), they displayed hyperactivity in the open field, while the adults in reverse showed a behavioral depression in this kind of test. Hyperactivity in the infantile age coincided with less grooming, or in other words, rats spent more time with locomotion at the expense of grooming activity. There is some evidence that the developing cholinergic system plays a role in the inhibitory function of hippocampus on motor behavior (2,6). The hyperactivity seen at the age of day 20 may partly be explained by such mechanisms. In the adult age, however, the lesioned animals showed signs of behavioral depression, since the start of exploration in the open field was markedly delayed and the rearings were much less than in controls. Damage to the septo-hippocampal system generally increases novelty-induced motility (18). In this respect, the behavioral depression observed in adult age after early postnatal cholinergic lesion may be unexpected. However, there are several new aspects concerning the specificity of the cholinergic lesion in the present experiment.

Firstly, the hippocampal cholinergic lesion occurred in the early period of postnatal brain development, which creates a condition for a variety of secondary plastic changes in this or in other neurotransmitter systems. Although our neurochemical findings argue against a significant homotypic cholinergic plasticity within the hippocampus, the histochemical results merit further discussion in this respect. The present results showed a strong cholinergic deafferentation of hippocampal principal cell layers, but relatively spared afferentation of regions containing large numbers of interneurons, i.e., the CA stratum oriens and the dentate hilus. As such, it may be concluded that the AF64A lesion more affected the cholinergic input to the hippocampal principal neurons compared to the interneurons. This conclusion is also strongly supported by our recent parallel study (23), showing a reduced expression of muscarinic receptors in the main cells, but not in the interneurons after 2 nmol AF64A ICV injection on PD 8. Thus the developmental AF64A lesion may result in a long-term lack of cholinergic activation of principal neurons, and persistent cholinergic activation of inhibitory (e.g.,

GABA-ergic) hippocampal interneurons. Therefore, the abnormal novelty-induced behavioral activation may be partly explained by a disproportional cholinergic arousal arriving at the main versus interneurons of the hippocampus.

Secondly, early postnatal anoxia has resulted in the same type of activity changes as observed here after AF64A lesion, i.e., short-term *hyperactivity* and adult behavioral *hypomotility* (31). For this reason, it is our future interest how perinatal hypoxia or anoxia influence the development of hippocampal cholinergic structures with special reference to differential influence on the various hippocampal cell types.

Finally, the postnatal AF64A lesion caused a severe deficit in learning a spatial task in a hole-board test. It has been well established that the hippocampal cholinergic system plays an important role in spatial learning requiring working memory [(5,13), among others]. A number of studies have shown that AF64A lesions in adult rats result in learning deficits in a series of behavioral tests including spatial learning tasks (1, 8, 13, 28, 43). Applied in the neonatal period, ICV AF64A (PD 1–4) was shown to produce impaired learning in passive and active avoidance tests (41). This latter study as well as our present results show that a cholinergic lesion during brain development has a long-lasting suppressive effect on learning ability. Since the present study showed the cholinergic lesion to be restricted to the hippocampus, we can conclude that maldevelopment of the hippocampal cholinergic system causes a persistent and serious impairment in spatial learning.

In the current experiments, the AF64A injection was performed at PD 8, i.e., in an age when the development of cholinergic afferentation reaches a critical stage. In this period, the expression of both biochemical and histological markers of cholinergic neurons accelerates (3,24), probably reflecting a massive advance in synapse formation. The presence of presynaptic cholinergic input is essential for the formation of synaptic integrity, since it has been found that the development of postsynaptic cholinergic structures, like muscarinic acetylcholine receptors, is seriously retarded after early postnatal cholinotoxin lesion in the hippocampus (23). In conclusion, the suppressed pre- and postsynaptic cholinergic functions in the hippocampus after the early postnatal AF64A lesion may be a major causal factor for the deficits seen in cognitive and mobility behavioral performance.

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